METHOD AND APPARATUS FOR DETERMINING THE AREA OR CONFLUENCY OF A SAMPLE

Field of the Invention

5   This invention relates to a method and apparatus for
       determining the area or confluency of a sample. The
       invention has particular application to generally
       transparent samples such as cells to enable the area or
       confluency of cells to be determined so that effects of
10   growth and confluency can be measured. However, it should
       be understood that the invention also has application to
       other sample types.

Background Art

15   Considerable difficulty can be experienced in measuring
       the area or confluency of some samples and, in particular,
       transparent samples. This is primarily due to the
       difficulty in determining where the boundary of the sample
       actually is so that the area or confluency of the sample
20   can be measured. Viable cells are transluscent objects
       that are difficult to visualise because there is usually
       little difference in contrast between cytoplasm and
       background. Cellular structures can be imaged and
       identified after staining or labelling, but this effects
25   the viability of the specimen. Visualising living cells
       in culture is particularly difficult due to their
       transparent nature, and also because there are inherent
       problems associated with imaging through plastic culture
       ware. It is important to be able to image living cells in
30   culture, not just for lineage maintenance, but also for
       evaluating the effects of growth intervention in vitro.

       Transparent viable unstained specimens, such as cells, can
       be visualised using optical phase microscopy which
35   enhances discrimination of the cells from their
       background. Optical phase microscopy was invented in the
       1930's by Fitz Zernike, and uses a phase plate to change

the speed of light passing directly through a specimen so
that it is half wavelength different from light deviated
by the specimen. This method results in destructive
interference and allows the details of the image to appear
5 dark against a light background. This visualisation of
the phase properties of a cell provides important
information about refractive index and thickness in phase
rich, amplitude poor transparent objects, which would
otherwise yield little information when examined using
10 bright field microscopy. Various implementations of phase
microscopy have been utilised in order to visualise
unstained, transparent specimens, including Dark Field,
Differential Interference Contrast, and Hoffman Modulation
Contrast. Although each of these methods allows enhanced
15 visualisation of transparent specimens, they all have
inherent problems, including cell edge distortion and the
generation of distinct halos at the edges of the cells,
making visual analysis difficult. More importantly, the
information provided by these techniques is useful for
20 qualitative analysis only.

Summary of the Invention
The object of the invention is to provide a method and
apparatus for enabling the area or confluency of a sample
25 to be determined, which does not destroy the sample, and
which also avoids the above-mentioned problems of prior
art optical techniques.

The invention provides a method of determining the area or
30 confluency of a sample, comprising:
        providing quantitative phase data relating to the
sample and background surrounding the sample;
        determining from the quantitative phase data the
boundary of the sample; and
35   determining the area within the boundary in order to
determine either the area of the sample or the confluency
of the sample.

Since quantitative phase data is used to obtain the area,
the sample is not destroyed, as may be the case if
staining is involved. Thus, growth patterns of the sample
5   can be measured over a predetermined time period if
desired by making subsequent measurements of the sample
over the predetermined time period. Furthermore, the
quantitative phase data avoids difficulties associated
with cell edge distortion and generation of halos, and
10   makes it much easier to identify the actual boundary of
the sample, thereby providing the determination of the
area or confluency of the sample.

Preferably the quantitative phase data is obtained by
15   detecting light from the sample by a detector so as to
produce differently focused images of the sample, and
determining from the different images the quantitative
phase data by an algorithm which solves the transport of
intensity equation so as to produce a phase map of the
20   sample in which the phase data is contained.

Most preferably the equation is solved in accordance with
the method described in International Patent Application
No. PCT/AU99/00949 in the name of Melbourne University,
25   and International Application No. PCT/AU02/01398 in the
name Iatia Imaging Pty Ltd. The contents of these two
International applications are incorporated into this
specification by this reference.

30   Preferably the step of determining the boundary of the
sample comprises forming a histogram of quantitative phase
data measurements of the sample and background, taking the
derivative of the histogram to thereby determine the point
of maximum slope of the histogram in the vicinity of the
35   boundary of the sample, and determining a line of best fit
on the derivative to obtain a data value applicable to the

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boundary so that data values either above or below the
determined data value are deemed within the sample.

Preferably the step of determining the area or confluency
5   comprises determining the area of confluency from the
number of data samples which are within the boundary.

In the preferred embodiment of the invention, each data
sample is applicable to a pixel of a detector and the area
10 of each pixel is known, so that from the known area of the
pixels and the number of pixels which register a data
value above or below the predetermined data value, the
area or confluency of the sample is determined.

15 The invention may also be said to reside in a method of
determining the area or confluency of a sample comprising:
        detecting light emanating from the sample by a
detector to form at least two images of the sample which
are differently focused to provide two sets of raw data;
20   from the two sets of raw data, determining a
quantitative phase map of the sample and its background;
        determining a boundary of the sample from individual
phase data values applicable to pixels of the detector
which are either above or below a determined pixel value;
25 and
        determining the area or confluency by multiplying the
pixel area by the number of pixels which are either above
or below the determined pixel value to thereby determine
the area or confluency of the sample.
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Preferably the pixel values are grey scale values and grey
scale values above a determined grey scale value are
deemed to be within the sample and are multiplied by the
pixel area to determine the area or confluency of the
35 sample.

Preferably the determined pixel value is determined by
identifying the greatest rate of change of grey scale
pixel values, thereby identifying the boundary of the
sample.

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Preferably the greatest rate of change is determined by
forming a histogram of grey scale values for all of the
pixels which detect the sample and its background,
determining the derivative of the histogram to provide a
10 graphical measure of the greatest rate of change of grey
scale values at various pixels, and determining the line
of best fit of the curve to determine the grey scale value
which defines the boundary of the sample so that all grey
scale values which are greater than the determined grey
15 scale value are deemed to be within the sample.

Preferably the raw data comprises at least one in focus
image of the sample and at least one out of focus image of
the sample.
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Most preferably the raw data comprises the in focus image
of the sample and one positively defocused image and one
negatively defocused image of the sample.

25 The invention provides an apparatus for determining the
area or confluency of a sample, comprising:
        a processor for:
        receiving quantitative phase data relating to the
sample and background surrounding the sample;
30   determining from the quantitative phase data the
boundary of the sample; and
        determining the area within the boundary in order to
determine either the area of the sample or the confluency
of the sample.
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Preferably the apparatus further comprises a detector for
producing differently focused images of the sample, and

the processor is for determining from the different images
the quantitative phase data by an algorithm which solves
the transport of intensity equation so as to produce a
phase map of the sample in which the phase data is

5   contained.

Preferably the processor determines the boundary of the
sample by forming a histogram of quantitative phase data
measurements of the sample and background, taking the

10   derivative of the histogram to thereby determine the point
of maximum slope of the histogram in the vicinity of the
boundary of the sample, and determining a line of best fit
on the derivative to obtain a data value applicable to the
boundary so that data values either above or below the

15   determined data value are deemed within the sample.

Preferably the processor determines the area or confluency
comprises determining the area of confluency from the
number of data samples which are within the boundary.

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In the preferred embodiment of the invention, each data
sample is applicable to a pixel of a detector and the area
of each pixel is known, so that the processor, from the
known area of the pixels and the number of pixels which

25   register a data value above or below the predetermined
data value, determines the area or confluency of the
sample.

The invention may also be said to reside in an apparatus

30   for determining the area or confluency of a sample
comprising:
        a detector for detecting light emanating from the
sample to form at least two images of the sample which are
differently focused to provide two sets of raw data;

35   a processor for determining from the two sets of raw
data, a quantitative phase map of the sample and its
background;

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the processor also determining a boundary of the sample from individual phase data values applicable to pixels of the detector which are either above or below a determined pixel value; and

5   the processor also determining the area or confluency by multiplying the pixel area by the number of pixels which are either above or below the determined pixel value to thereby determine the area or confluency of the sample.

10   Preferably the pixel values are grey scale values and grey scale values above a determined grey scale value are deemed to be within the sample and are multiplied by the pixel area to determine the area or confluency of the sample.

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Preferably the determined pixel value is determined by identifying the greatest rate of change of grey scale pixel values, thereby identifying the boundary of the sample.

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Preferably the greatest rate of change is determined by the processor forming a histogram of grey scale values for all of the pixels which detect the sample and its background, determining the derivative of the histogram to

25   provide a graphical measure of the greatest rate of change of grey scale values at various pixels, and determining the line of best fit of the curve to determine the grey scale value which defines the boundary of the sample so that all grey scale values which are greater than the

30   determined grey scale value are deemed to be within the sample.

Preferably the raw data comprises at least two defocused images equally spaced either side of the focus.

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Most preferably the raw data comprises the in focus image
of the sample and one positively defocused image and one
negatively defocused image of the sample.

5   The invention provides a computer program for determining
the area or confluency of a sample from providing
quantitative phase data relating to the sample and
background surrounding the sample, comprising:
        code for determining from the quantitative phase data
10   the boundary of the sample; and
        code for determining the area within the boundary in
order to determine either the area of the sample or the
confluency of the sample.

15   Preferably the quantitative phase data is obtained by
detecting light from the sample by a detector so as to
produce differently focused images of the sample, and the
program includes code for determining from the different
images the quantitative phase data by an algorithm which
20   solves the transport of intensity equation so as to
produce a phase map of the sample in which the phase data
is contained.

Preferably the code for determining the boundary of the
25   sample comprises code for forming a histogram of
quantitative phase data measurements of the sample and
background, code for taking the derivative of the
histogram to thereby determine the point of maximum slope
of the histogram in the vicinity of the boundary of the
30   sample, and code for determining a line of best fit on the
derivative to obtain a data value applicable to the
boundary so that data values either above or below the
determined data value are deemed within the sample.

35   Preferably the code for determining the area or confluency
comprises code for determining the area of confluency from
the number of data samples which are within the boundary.

In the preferred embodiment of the invention, each data sample is applicable to a pixel of a detector and the area of each pixel is known, so that from the known area of the
5   pixels and the number of pixels which register a data value above or below the predetermined data value, the area or confluency of the sample is determined.

The invention may also be said to reside in a computer
10   program for determining the area or confluency of a sample by detecting light emanating from the sample by a detector to form at least two images of the sample which are differently focused to provide two sets of raw data, comprising:
15   code for determining from the two sets of raw data, a quantitative phase map of the sample and its background;
        code for determining a boundary of the sample from individual phase data values applicable to pixels of the detector which are either above or below a determined
20   · pixel value; and
        code for determining the area or confluency by multiplying the pixel area by the number of pixels which are either above or below the determined pixel value to thereby determine the area or confluency of the sample.
25

Preferably the pixel values are grey scale values and grey scale values above a determined grey scale value are deemed to be within the sample and are multiplied by the pixel area to determine the area or confluency of the
30   sample.

Preferably the determined pixel value is determined by code for identifying the greatest rate of change of grey scale pixel values, thereby identifying the boundary of
35   the sample.

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Preferably the greatest rate of change is determined by
code for forming a histogram of grey scale values for all
of the pixels which detect the sample and its background,
code for determining the derivative of the histogram to
5   provide a graphical measure of the greatest rate of change
of grey scale values at various pixels, and code for
determining the line of best fit of the curve to determine
the grey scale value which defines the boundary of the
sample so that all grey scale values which are greater
10 than the determined grey scale value are deemed to be
within the sample.

Preferably the raw data comprises at least one in focus
image of the sample and at least one out of focus image of
15 the sample.

Most preferably the raw data comprises the in focus image
of the sample and one positively defocused image and one
negatively defocused image of the sample.
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Brief Description of the Drawings
A preferred embodiment of the invention will be described,
by way of example, with reference to the accompanying
drawings in which:
25   Figure 1 is a view of an apparatus embodying the
invention;
        Figure 2 is a view of an image of a sample as formed
on a detector used in the embodiment of Figure 1;
        Figure 3 is a histogram of sample values used to
30 identify a boundary of the sample in the image of Figure
2;
        Figure 4 is a graph showing the derivative of the
histogram curve of Figure 3.

35 Detailed Description of One Embodiment of the Invention
With reference to Figure 1, an apparatus 10 is shown for
determining the area or confluency of a sample. The

apparatus 10 comprises a detector 12 such as a charge
coupled device type camera or the like. The camera 12, as
is well known, is formed from a number of pixels generally
in a rectangular array.

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A sample stage 14 is provided for holding a sample such as
a cell in a transparent dish or on a slide, etc. A light
source 16 is provided for providing light. The reference
to light used in the specification should be understood to
10   mean visible as well as non-visible parts of the
electromagnetic spectrum, and also particle or acoustic
radiation.

The light from the sample 16 passes through conditioning
15   optics schematically shown at 20 so as to form a beam of
light 22 which passes through the sample S and which is
detected by the detector 12.

In order to form a quantitative phase map of the sample S
20   and its surrounding background, three images of the sample
are produced at different focuses. The first image is an
in focus image at the position of the stage 14 shown in
Figure 1. The second image is a positively defocused
image at the position 14', and the third image is a
25   negatively defocused image at the position 14''. The raw
data obtained by these three images is used in an
algorithm to solve the transport of intensity equation so
that quantitative phase data relating to the sample and
the background surrounding the sample is obtained. The
30   algorithm used to form the quantitative phase map is
disclosed in the aforementioned International
applications, and therefore will not be repeated in this
specification. It should be understood that whilst this
method of forming the quantitative phase map is preferred,
35   other techniques for providing the quantitative phase map
of the sample may also be used.

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The quantitative phase map is produced in processor 40, which is connected to the detector 12, and a phase image of the sample S may be viewed on a monitor 50 connected to the processor 40.

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Figure 2 is a view of the image which may be obtained which shows the sample S and its surrounding background, which is most conveniently white. The algorithm which solves the transport of intensity equation therefore is

10   able to provide a quantitative phase measure at each pixel of the detector 12, applicable to the sample S and its surrounding background. If desired, the background of the sample S may be masked by a mask M as shown in Figure 2, so that spurious events such as dust or the like which may

15   be in the background is reduced to a minimum. Each of the pixels of the detector 12 within the mask M is therefore provided with a grey level value of from between 0 and 255, which is indicative of the quantitative phase measurement at that pixel of the sample S and its

20   surrounding background within the mask M.

Once the quantitative phase data for each pixel in the detector 12 has been determined, a histogram as shown in Figure 3 of the grey scale values for the pixels can be

25   created. Typically, the histogram will be similar to that shown in Figure 3, in which the surrounding background has a very low grey scale value V applicable to "black light" or zero phase retardation of the light as it passes by the sample S. It should be understood that usually this grey

30   scale image is seen as a white on black image so that the background area surrounding the sample S is typically black and the image of the sample appears white. However, if the nature of the system is such that the background area is thicker and has more phase retardation than the

35   sample, then the opposite will be the case and, furtherstill, if desired, the usual image could be inverted so that the background appears white and the

sample appears as a darker or black contrast. The grey
scale value within the sample S will increase because of
phase retardation as the light passes through the sample
S, thereby tending to provide a lighter colour and
5   therefore a higher grey level value V. Typically, the
mean value of the sample S may be, for example, a grey
level of 175 as shown in Figure 3.

The boundary of the sample S will be indicative of the
10   location where there is the greatest change between
adjacent pixel values. The reason for this is that
outside the boundary, the background will provide no
retardation, and therefore a very low grey level value of,
for example, 20. At the boundary, and within the sample
15   S, the pixel value will be much higher. Thus, by
determining the point on the histogram which is in the
area of the sample boundary, and which shows the greatest
rate of change, an indication of the grey level value at
the boundary of the sample S can be obtained. In order to
20   determine the greatest rate of change, the derivative of
the histogram function in the vicinity of the boundary is
determined. This is also performed by the processor 40.

A user can identify the likely location of the boundary by
25   viewing the histogram in Figure 3. The part of the curve
marked A in Figure 3 will be clearly attributable to the
large number of pixels which show background and will
generally have a very low grey scale value because of no
phase retardation by the sample S. The part of the curve
30   marked B in Figure 3 will be recognised to be in the
boundary region, and the derivative function can typically
be taken of the part of the curve between the points, for
example, C and D in Figure 3. The turning point E of the
graph will be the part of the derivative which crosses the
35   X axis in Figure 4 and the part of the curve G in Figure 4
will be the line which identifies the grey level value V
in Figure 4 attributable to the boundary of the sample S.

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Thus, by forming a line L in Figure 4 of best fit to the
part of the curve G, the grey scale value of the pixels
which identify the boundary can be determined. In the
example of Figure 4, the grey scale value is 160.

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The area or confluency of the sample S is therefore
determined by determining the number of pixels which
provide a grey scale value of 160 or greater, and
multiplying the number of such pixels by the area of each
10   pixel. This will therefore provide the area of the sample
S or the confluency of the sample if the sample is a
number of cells which are joined together.

Examples of the invention are given below.
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Airway smooth muscle cells were obtained by collagenase
and elastase digestion from bronchi of lung transplant
resection patients. Cultures were maintained in phenol
red-free DMEM with 10% FCS, supplemented with 2mM L-
20   glutamine, 100U/ml penicillin-G, 100μg/ml streptomycin and
2μg/ml amphotericin B. Cells were passaged weekly at a 1:4
split ratio by exposure to 0.5% trypsin containing 1mmol/L
EDTA. For experiments measuring confluency, cells were
seeded onto plastic culture dishes at 2.5 x 10^4 - 4 x 10^4
25   cells/well in media as above. A period of 24 hours was
allowed for adherence of cells to the culture dish and
measurements were then obtained daily with a media change
after 3 days.

30   <u>Example 1</u>
Bright field images were captured using a black and white
1300 x 1030 pixel Coolsnap FX CCD camera (Roper
Scientific) mounted on a Zeiss Axiovert 100M inverted
microscope utilising a Zeiss Plan-Neofluar (x10, 0.30 NA)
35   objective. To ensure optimal specimen illumination, Köhler
illumination conditions were established for each optical
arrangement (condenser and objective alignment and

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condenser stop at 70% field width). In order to calculate
the phase map, one in-focus, and equidistant positive and
negative de-focus images were acquired, using a defocus
distance of zz μm in this instance. This was achieved
5   using a piezoelectric positioning device (PiFoc, Physik
Instrumente, Karlsruhe, Germany) for objective
translation.   Bright field images were subsequently
processed to generate phase maps using QPm software (v2.0
IATIA Ltd, Australia). The phase map generation, based on
10   the set of three bright field images captured, involved
software-automated calculation of the rate of change of
light intensity between the three images[6]. In addition
to the set of images obtained for phase map calculation,
for each specimen an image using conventional optical
15   phase techniques was also acquired (Plan-Neofluar, x10, NA
0.30) in order that a comparison of calculated and optical
phase imaging techniques could be performed. An example
and comparative view of the three different image types
(bright field, phase map and optical phase) are shown in
20   Fig 5. The lack of structural detail observable in the
bright field image is notable when compared to the two
phase images (Fig 5A). The distinct cell boundary
definition achieved using the QPm software calculated
phase map (Fig 5B) when compared with an optically derived
25   phase image (Fig 5C) is also apparent.

Phase map images were analysed to evaluate confluency and
to measure the growth of the cultured muscle cells over
the period of 92 hours. Reproducible location of a
30   reference point within the culture dish was achieved using
a mark on the base of the culture plate and by reference
to the gradation scale on the microscope stage. This
enabled measurements of the same area of cells (those in
the field surrounding the centred reference point) over
35   the extended time period at 24 hour intervals.

Culture plates were set up so that parallel measurements

of confluency and determination of cell number could be
performed at each time interval. Following phase image
capture, cells were lifted from the culture substrate by
exposure to trypsin (0.5% v/v containing 1 mmol EDTA) and

5   counted using standard haemocytometry. To ensure uniform
growth rates across the 6 well plates, all wells were
seeded at the same density, from the same cell passage
type, and were exposed to identical incubation conditions.
One well of the six well plate was repeatedly imaged for

10   daily confluency measurement with the remaining five wells
harvested one per day for cell number determination. The
relationship between cell growth measurements obtained by
confluency measurement of phase maps and by
haemocytometric cell counting methods was estimated.

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Inspection of the images presented in Figure 5 illustrates
the difficulties encountered in visualising cultured cell
monolayers under bright field conditions. The cellular
outlines and processes are barely discernible in Figure

20   5A, despite the optimised Koehler illumination conditions.
In Figure 5B, as is typically observed, the calculated
phase map exhibits a much enhanced dynamic contrast range.
The optical phase image of the same field presented in
Figure 5C offers somewhat improved contrast relative to

25   the bright field image. This is particularly accentuated
(and somewhat distorted) at the cell boundaries, but the
optical phase view provides less useful contrast between
the internal cellular and non-cellular image features.

30   Phase maps (ie Fig 5B) were analysed (using the QPm
software image analysis tools) to construct pixel
intensity histograms (Fig 6A) to identify phase shift
characteristics associated with cellular structures.
Scrutiny of numerous phase map histograms indicated that

35   the initial portion of the steepest gradient of the
histogram could be used to reproducibly demarcate cellular
material from extracellular material. A linear function

was fit to the ascending portion of the derivative of the intensity histogram (Fig 6B) and extrapolated to the x-axis to obtain the threshold grey level at which segmentation of cellular from non-cellular material could
5   be achieved using the phase map (Fig 6C). This novel calculation provides an entirely non-subjective technique of image segmentation for cell delineation. The extrapolated threshold value was then utilised to construct a binary image (Image-Pro Plus software v3.0
10   Media Cybernetics, USA) representing demarcation of cellular material from non-cellular material in the phase image (see Fig 6D). The binary map generated by these segmentation manipulations is simply used to sum the quantity of 'black' delimited cellular material on the
15   culture plate as a measure the confluency of the culture, expressed as a percentage of the total field area examined. (% section area). For the culture used as a 'case' image analysis presented in Figures 5 and 6, this value was 5.68%, a value typical for cultures at about
20   20hr post seeding under these conditions.

An 8 bit image (grey scale representing values ranging from 0-255) as found to be optimal for the segmentation procedure summarised above. The analysis was also
25   undertaken using a 12 bit image to increase the contrast range available, and potentially to improve the precision of determination of the threshold point. However, an increase in noise in the 12 bit image histograms was generally observed to offset any improvement in the
30   determination of the threshold grey-level.

This analysis procedure allows for an accurate and non-biased calculation of the threshold point with which to distinguish cells from background. Of crucial importance
35   in achieving a successful thresholding outcome in this process is the quality of data available in the phase map where haloing and cell edge distortion is suppressed

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allowing for accurate cell delineation. When the same
analysis procedure was attempted with an image captured
using conventional optical phase techniques, a reliable
outcome could not be achieved (Figure 7). The reduced

5   difference in contrast between cellular and non-cellular
material in the optical phase image renders the curve-
fitting procedure unstable, while the effect of uneven
illumination intensity in the optical image produces
regional variation in the thresholding process. Inspection

10   of the derivatives generated from the optical phase image
intensity histograms revealed that these plots are
somewhat more complex than those extracted from the phase
maps and exhibit multiple peaks (Figure 7A). Thus the
process of intercept extrapolation cannot be easily

15   applied to these plots and it is not possible to employ a
non-subjective thresholding method. The difficulties
associated with segmentation of optical phase images are
exemplified in Fig 7B, where it is apparent that the
binary image produced by thresholding incorporates

20   extracellular regions at the top left of the image (Fig
3D) and fails to delineate boundaries at other locations
in the lower portion of the image. The combined effect of
this lack of field uniformity and cell delineation
difficult, markedly over-estimates the confluency status

25   of this culture specimen by more than 2-fold when compared
to the phase map determination using similar thresholding
methods.

Phase-map thresholding and segmentation techniques were
30   applied to measure the progressive increase in confluency
of HASM cell cultures from several different patient cell
lines. Following re-passaging and seeding at standardized
density, culture growth was tracked by repeated imaging
over a 92 hour time period. As shown in Figure 8A, an
35   approximately linear growth response was observed over
this period, with the degree of confluency increasing from
about 8 % at 24 hours to around 17 % after 92 hours.

Figure 8B illustrates the correlation between the
quantitative phase calculated culture confluency and cell
number determined by haemocytometry for the same culture
5   wells throughout this growth period for the three lines
tracked in Figure 5A. A high degree of correlation (r^2 =
0.95) is observed between these two growth measures.
These findings indicate that in circumstances where
proliferative growth is conventionally (and destructively)
10   assessed by cell harvest and counting, the use of *in situ*
QPM imaging methods provides a reliable and non-
destructive surrogate.